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(54) Title: PRODUCT AND METHOD FOR IMPROVING KERATINOCYTE ADHESION TO THE DERMIS

(57) Abstract

A purified protein kalinin is disclosed that provides adhesion between epidermal keratinocytes and the underlying dermis. Purified kalinin localizes to the anchoring filaments of basement membranes of human subepithelial skin, trachea, esophagus, cornea and amnion when exposed to monoclonal antibody BM165. The protein has a molecular weight of approximately 400-440kDa and separates on Western blots into fragments of 165kDa, 155kDa, 130kDa and 105kDa when its disulfide bonds are reduced. The epitope of BM165 is identified on the 165kDa fragment when the blots are probed with BM165. The protein has a rotary shadow image which reveals an asymmetric 170nm long rod having two globules at a first end and a single globule at a second end of the rod. A method is also disclosed for improving adhesion of transplanted keratinocytes to an underlying substrate, such as the human dermis, by optimizing the production of kalinin from cultured keratinocytes, or providing an exogenous source of kalinin between the keratinocytes and substrate.

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PRODUCT AND METHOD FOR IMPROVING KERATINOCYTE ADHESION TO THE DERMIS

BACKGROUND OF THE INVENTION

1. Field of the Invention

This invention concerns a basement membrane protein useful in adhering keratinocytes to the dermis. More specifically, this invention concerns a method of using this protein to enhance the success of skin transplants.

2. General Background of the Invention

The use of cultured epidermal grafts (keratinocyte grafts) to treat patients with life-threatening burns was first reported by O'Conner et al, 1981; The Lancet 1:75-78. Small skin biopsy specimens from burn patients were cultured in vitro, and the cultured autografts were placed on full thickness wounds on the arms of burn patients. The cultured keratinocytes successfully grew to cover the wounds in six weeks. Subsequent attempts have been made to improve this method by modifying it to grow keratinocytes in serum-free medium. Others have suggested using composite cadaver skin allografts resurfaced with autologous cultured keratinocytes. Attempts have also been made to use different backing materials for the cultured cells or to vary the keratinocyte culture methodology. The results of cultured keratinocyte transplants, however, have often been disappointing.

One of the most useful applications for keratinocyte grafts has been in patients with burns damaging more than half of the body surface. Such patients have insufficient donor sites to provide enough split skin thickness grafts to resurface the area of the burn after surgical excision. Unfortunately, the results of keratinocyte autografting in these circumstances have been variable and disappointing. Cultured epidermal grafts have been found to be significantly more fragile

than normal skin and more prone to blistering. Woodley et al, 1988; JAMA 259:2566-2571. Some researchers have suggested that an abnormality in one or more connective tissue components within the autografts might explain the altered epidermal-dermal adherence observed clinically. The identity of that component, however, has remained obscure.

It is an object of this invention to identify and provide a therapeutically useful form of a connective tissue component that provides epidermal-dermal adherence.

It is another object of this invention to use such a therapeutically useful substance to enhance the adhesion of transplanted cultured keratinocytes to an underlying substrate, such as a mammalian or human dermis.

These and other objects of the invention will be understood more clearly by reference to the following detailed description.

SUMMARY OF THE INVENTION

The foregoing objects have been achieved by identification and production of a purified new protein which is present in the anchoring filaments of the basement membranes of human subepithelial skin, trachea, esophagus, cornea and amnion. This new protein, which has been named kalinin by its discoverers, has been found to provide adhesion between the human dermis and epidermis. Kalinin has a molecular weight of 400-440kDa, and separates on Western blots into fragments of 165kDa, 155kDa, 140kDa, and 105kDa after its disulfide bonds are reduced. The epitope of monoclonal antibody BM165 is identified on the 165kDa fragment when the blots are probed with BM165. Rotary shadow imaging of the protein reveals an asymmetric 170nm long rod having two globules at a first end and a single globule at the opposite end. Kalinin has been found to be absent in the dermal-

epidermal junction of humans with diseases such as junctional epidermolysis bullosa, in which the epidermis separates from the underlying dermis.

The invention also includes a method of improving adhesion of transplanted keratinocytes to an underlying substrate by providing an amount of kalinin between the keratinocytes and substrate which is greater than the amount produced naturally by keratinocytes. This increased amount of kalinin can be supplied by applying exogenous purified kalinin to the substrate or the basal surface of keratinocytes prior to placing the confluent keratinocyte culture on a graft site. Alternatively, the cultured keratinocytes can be induced to increase their basal levels of kalinin production to supra-physiologic levels by using growth promoters such as cytokines. Alternatively, the cultured keratinocytes are monitored to determine whether they are actively producing kalinin, and the keratinocytes are transplanted to a substrate before active kalinin production declines significantly.

BRIEF DESCRIPTION OF DRAWINGS

FIG. 1A is a photomicrograph showing the indirect immunofluorescent localization of the BM165 antigen in human foreskin.

FIG. 1B is a photomicrograph similar to FIG. 1A wherein the frozen section was stained with media from unfused myelomas.

FIG. 2A is a photomicrograph of the dermal-epidermal junction in human skin showing the ultrastructural features of this region, the bar representing a length of 100nm.

FIG. 2B is a photomicrograph similar to FIG. 2A showing localization of BM165 monoclonal antibody to the anchoring filaments of the dermal-epidermal basement membrane.

FIG. 2C is a photomicrograph similar to FIG. 2B, at a lower magnification, showing BM165 labeling along a continuous stretch of intact skin, the bar representing a length of 200nm.

FIG. 2D is a photomicrograph similar to FIG. 2C showing BM165 labeling along the basement membrane in a region where the antibody has induced epidermal detachment.

FIG. 3A is a photomicrograph of a confluent keratinocyte culture stained with BM165, the small bar indicating a length of 20nm.

FIG. 3B is a photomicrograph similar to FIG. 3A wherein the confluent keratinocyte culture was stained with control media.

FIG. 3C is a photomicrograph similar to FIG. 3A wherein the transmission electron micrograph section is taken through the cell layer parallel to the culture substrate, the black bar indicating a length of 20nm.

FIG. 3D is a photomicrograph similar to FIGS. 3A-C in which the cells are removed from the substrate with EDTA before staining with BM165.

FIG. 4A is a photomicrograph of a continuous subcellular matrix in a keratinocyte culture that was grown to near confluency, then washed with PBS and incubated with BM165 monoclonal antibody followed by 5nm gold conjugated secondary antibody prior to fixation.

FIG. 4B is a photomicrograph similar to FIG. 4A in which the keratinocytes were grown to near confluency and fixed immediately without BM165 staining.

FIG. 4C is a scanning electron micrograph of cells prepared as in FIG. 4A.

FIG. 4D is a scanning electron micrograph of a confluent culture prepared as in FIG. 4B.

FIG. 5A is a photomicrograph of keratinocytes grown to 75-80% confluency, then washed and treated with PBS and photographed 10 minutes after treatment; the bar represents 20μm.

FIG. 5B is a photomicrograph similar to FIG. 5A in which the cells were photomicrographed 60 minutes after PBS treatment.

FIG. 5C is a photomicrograph similar to FIG. 5A in which the cells were washed with PBS and treated with 50μg/ml BM165 mAb then photographed after 10 minutes.

FIG. 5D is a photomicrograph similar to FIG. 5C, 60 minutes after exposure to BM165 mAb.

FIG. 5E is a photomicrograph similar to FIG. 5A in which the cells were washed with PBS and treated with 10mM EDTA, photographed 10 minutes after treatment.

FIG. 5F is a photomicrograph similar to FIG. 5E, but taken 60 minutes after treatment with EDTA.

FIG. 6A is a photomicrograph of cultured keratinocytes that were exposed to the BM165 antibody after 6 hours, the bar representing a length of 50μm.

FIG. 6B is a photomicrograph similar to FIG. 6A in which the cultured keratinocytes were exposed to the BM165 antibody after 24 hours in culture.

FIG. 6C is a photomicrograph similar to FIG. 6A in which the keratinocytes were exposed to BM165 after 48 hours in culture.

FIG. 6D is a photomicrograph similar to FIG. 6B illustrating substrate labeling along the paths of migrating keratinocytes.

FIG. 6E is a figure similar to FIG. 6D.

FIG. 7 is a Western blot showing an electrophoretic analysis of the BM165 antigen isolated from keratinocyte culture medium.

FIG. 8A is a photograph showing rotary shadow analysis of the BM165 antigen following affinity purification, the bar representing a length of 100nm.

FIG. 8B is a higher magnification photograph of the rotary shadow images of FIG. 5A.

FIG. 8C is another high magnification view of the rotary shadow analysis of FIG. 8A.

FIG. 9 is a schematic diagram of the ultrastructure of the basement membrane region at the dermal-epidermal junction of human skin.

FIG. 10 is a graph of the amount of kalinin immunprecipitated with antibody BM165 from keratinocytes radiolabed during the indicated times of culture.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

The ultrastructure of the basement membrane at the epidermal-dermal junction is shown schematically in FIG. 9, which depicts the lower part of a basal keratinocyte 10 having a plasma membrane 12 that seats on a lamina lucida 14, subadjacent lamina densa 16, and dermis 18. A hemidesmosome 20 is depicted at the basal portion of keratinocyte 10 on plasma membrane 12. Tonofilaments 22 insert into the hemidesmosome 20 and extend into the cytoplasm. Anchoring filaments 24 arise from the plasma membrane beneath the attachment plaque of hemidesmosome 20. The filaments traverse the lamina lucida 14 and connect the basal plasma membrane 12 with the lamina densa 16, and are most numerous in the region of the hemidesmosome. Anchoring fibrils 26, in contrast, are short curved structures, with an irregularly spaced cross banding of their central portions, which fan out at either end. The distal part of fibrils 26 inserts into the lamina densa while the proximal part terminates in the papillary dermis or loops around to merge into the lamina densa. The present invention concerns a protein associated with the anchoring filaments 24, which performs an important function in adhering the dermis to the epidermis.

The ultrastructure of the anchoring fibril network suggests that it secures the basement membrane to the underlying dermis (Susi et al. 1967; J. Cell Biol. 34:686-690. Kawanami et al, 1978; Am. J. Pathol. 92:389-410). This hypothesis is supported by observations that individuals with recessive dystrophic epidermolysis

bullosa lack anchoring fibrils (Briggaman et al, 1975b; J. Invest. Dermatol. 65:203-211. Leigh et al, 1988; J. Invest. Dermatol. 90:612-639. Bruckner-Tuderman et al, 1989; J. Invest. Dermatol. 93:3-9), and suffer from spontaneous separation of the epidermal basement membranes from the subadjacent stroma.

The molecular heterogeneity of the dermal-epidermal junction is reflected by the presence of several glycoproteins localized to this zone. Hemidesmosomes, for example, contain several proteins having M_r values from 165,000-240,000. (Mutasim et al, 1989; J. Invest. Dermatol. 92:225-230. Westgate et al, 1985; J. Invest. Dermatol. 81:149-153. Regnier et al, 1985; J. Invest. Dermatol. 85:187-190. Jones et al, 1986; Cell Motil. Cytoskel. 6:560-569. Stanley et al, 1984; J. Invest. Dermatol. 82:108-111. Labib et al, 1986; J. Immunology 136:1231-1235. Mueller et al, 1989; J. Invest. Dermatol. 92:33-38). An integrin $\alpha 6\beta 4$ has also been recently localized to the external region of the hemidesmosome by Stepp et al, 1990; Proc. Nat. Acad. Sci. (USA) 87:8970-8974. The anchoring fibrils themselves include lateral unstaggered aggregates of type VII collagen described by Sakai et al, 1986a; J. Cell Biol. 103:1577-1586, and Lunstrum et al, 1987; J. Biol. Chem. 262:13706-13712.

In addition to these known proteins, several antibodies have been found which recognize antigens unique to the dermal-epidermal junction. An example is the murine monoclonal antibody 19-DEJ-1 which recognizes an unknown antigen that is absent from the basement membrane of skin from patients with epidermolysis bullosa (Fine, 1988; Arch. Dermatol. 124:713-717). The antigen detected by 19-DEJ-1 is unknown and only partially characterized.

The structural relationship among proteins of the hemidesmosomes, the basement membrane constituents, and type VII collagen in anchoring fibrils has not been clearly elucidated. Some researchers have suggested an

association between anchoring fibrils and the hemidesmosomes based on ultrastructural analyses in which hemidesmosomes are found to regenerate only on sites along denuded basement membranes directly over anchoring fibrils (Gipson, 1983; J. Cell Biol. 97:849-857. Susi et al, 1967; J. Cell Biol. 34:686-690. Ellison et al, 1984; J. Cell Sci. 72:163-172). Hemidesmosomes and anchoring fibrils have also been observed to appear simultaneously during fetal development and wound healing (Gipson et al, 1988; Dev. Biol. 126:253-262. Smith et al, 1988; J. Invest. Dermatol. 90:480-485). Published models for the structure of anchoring fibrils predict that direct contact between type VII collagen and the hemidesmosome is unlikely because the NC-1 domain of type VII collagen has a diameter of only 50nm and is not large enough to span the entire basement membrane which is wider than 50nm. Hence, other proteins likely link type VII collagen to the hemidesmosomal proteins. (Lunstrum et al, 1987; J. Biol. Chem. 262:13706-13712. Keene et al, 1987; J. Cell Biol. 104:611-621. Bächinger et al, 1990; J. Biol. Chem. 265:10095-10101).

The present inventors have described a protein associated with anchoring filaments. This protein is further characterized by ultrastructural location and tissue distribution. The protein has been purified and its fibrous conformation determined by shadow imaging. Finally, this newly purified protein is shown to be necessary for the in vitro attachment of keratinocytes to plastic or glass substrates and to the basement membrane in vivo.

Source of Immunogen

Kalinin is localized using the monoclonal antibody BM165. The BM165 immunogen was derived from an extract of human amnion, prepared as follows. Collagenase

extraction and purification of the NC-1 globular domain of type VII collagen from human amnion has been described previously (Bächinger et al, 1990; J. Biol. Chem. 265:10095-10101). During one step of this purification, the extract is incubated with DEAE-cellulose (DE52, Whatman) in a low salt buffer (2 M urea, 25 mM NaCl, 5 mM EDTA and 50 mM Tris-HCl, pH 7.8). This unbound fraction was used in the further purification of the NC-1 domain. The DEAE was washed with an equal volume of buffer containing 0.2 M NaCl and the eluted material was isolated after centrifugation (17,000 x g, 60 min). The sample was concentrated 10 fold by ammonium sulfate precipitation (50% saturation) and equilibrated in PBS by dialysis. The resulting complex mixture of proteins served as immunogen in the preparation of hybridomas.

Keratinocyte Cell Culture

Human foreskin keratinocytes were prepared according to the published procedures of Boyce et al, 1985; J. Tiss. Cult. Meth. 9:83-93, which are incorporated by reference. Cells were grown in Keratinocyte Growth Medium containing 0.15 mM $CaCl_2$ and subcultured according to the manufacturer's instructions (Clonetics). For most immunocytochemical experiments, first or second passage cells were grown in glass or plastic chamber slides (Lab-Tek) or on glass cover slips to approximately 80% confluency. For large scale collection, spent media cells were grown in 150 cm^2 tissue culture dishes and fed three times per week with 15 ml fresh media.

Affinity Purification of the BM165 Antigen

Media collected from growing keratinocytes was clarified by centrifugation (2,000 x g, 10 min.) and endogenous protease activity was minimized by the addition of EDTA, PMSF and N-ethylmaleimide to final concentrations

of 5 mM, 50 uM, and 50 uM, respectively. The media was sterilized by filtration and either processed immediately or stored frozen at -20°C until use. The BM165 mAb was conjugated to CNBr-activated Sepharose 4B (Pharmacia LKB Inc.), at 1 mg of antibody per ml of resin, as described by the manufacturer. Keratinocyte media (1-2 liters) was passed through a 15 ml antibody column and the column was washed with PBS. The antigen was eluted with 1 M acetic acid and fractions were monitored for absorbance at 280 nm. Pooled fractions were treated with diisopropylfluorophosphate (5 ug/ml) and dialyzed into appropriate buffers for further analysis. To perform SDS-PAGE, samples were separated on 3-5% gradient gels before reduction and on 5% gels after reduction with β-mercaptoethanol. In addition to high molecular weight pre-stained standards (Biorad), the disulfide bonded type VII collagen NC-1 domain (Mr 450,000), reduced NC-1 (Mr 150,000) and reduced fibrillin (Mr 350,000, Sakai et al, 1986b) were used in determining Mr scales.

Tissue Preparation

Enbloc immunolocalization of antigens was performed as previously described by Keene et al, 1987; J. Cell Biol. 104:611-621, with some modification. Human neonate foreskin collected shortly after circumcision was cut into 0.5 mm x 1 mm blocks, all including epithelium, and washed for two hours in phosphate buffered saline (PBS), pH 7.4 at 4°C, rinsed in several changes of PBS over 6 hours, then incubated in 1 nm gold conjugated secondary antibody (Janssen Life Sciences Products, Piscataway, N.J.) diluted 1:3 in PBS containing 1.0% BSA overnight at 4°C. Following washing, tissues were submersed in ice cold silver intensification solution (Janssen Life Sciences Products, Piscataway, N.J.) for 15 minutes, then rapidly warmed to room temperature. After allowing silver to precipitate upon the 1 nm gold

particles for seven minutes at room temperature, the tissues were rinsed several times over 15 minutes with water, then with 0.1 M cacodylate buffer at pH 7.4. Tissues were finally fixed in 0.1 M cacodylate buffered 1.5%/1.5% glutaraldehyde/paraformaldehyde pH 7.4, dehydrated in a graded series of ethanol dilutions, exposed to propylene oxide, and embedded in Spurrs epoxy. Control antibodies used included those recognizing elastin (produced and provided by Dr. Lynn Sakai), collagen type IV (Sakai et al, 1982; Am. J. Pathology 108:310-318), and collagen type VI (Keene et al, 1988; J. Cell. Biol. 107:1995-2006). One sample of skin was fixed for 30 minutes in ice cold acetone, rinsed in buffer, further fixed in 3%/3% aldehydes and 1% OsO_4, then dehydrated in acetone prior to embedding in Spurrs epoxy in order to demonstrate the presence of anchoring filaments (FIG. 2A).

Electron Microscopic Examination

For examination of normal cell ultrastructure prior to antibody treatment, human keratinocyte cultures were grown on glass coverslips and fixed in 0.1 M cacodylate buffered 1.5%/1.5% glutaraldehyde/paraformaldehyde, 1.0% OsO_4, dehydrated in a graded ethanol series, then either embedded directly in Spurrs epoxy for transmission electron microscopy (TEM), or critical point dried and sputter coated for scanning electron microscopy (SEM) as previously described (Keene et al, 1988; J. Cell. Biol. 107:1995-2006). TEM immunoelectron microscopy was performed on keratinocytes grown on 8 well permanox culture flasks using an identical protocol as that described above for tissues, except that the incubation time in primary antibody was for four hours at room temperature, the secondary antibody was conjugated to 5 nm gold and diluted 1:3 in BSA buffer (20 mM Tris-HCl, 0.9% NaCl, 1 mg/ml BSA, 20 mM NaN_3), and the silver intensification procedure was omitted. Keratinocytes

grown on glass coverslips and observed by SEM following exposure to antibody were treated identically, except they were critical point dried from liquid CO_2 following dehydration in ethanol.

For routine TEM examination, 60 - 90 nm thick sections were cut on a Reichert ultramicrotome using diamond knives and examined using a Philips 410 LS operated at 60 KV following contrasting in uranyl acetate and Reynolds lead citrate (Reynolds, 1963; J. Cell Biol. 17:208-215). For routine SEM examination, samples were sputter coated with a minimum amount of Gold-Palladium and observed in the upper stage of a scanning EM (model DS130; International Scientific Instruments, Inc., Milpitas, CA) operated at 10 KV, using a spot size of 3 - 10 mn.

Other Techniques

Methods including Western blotting, rotary shadow analysis and length measurements have been detailed elsewhere (Morris et al, 1986; J. Biol. Chem. 261:5638-5644. Lunstrum et al, 1986; J. Biol. Chem. 261:9042-9048. Bächinger et al, 1990; J. Biol. Chem. 265:10095-10101).

Rotary shadowing of molecules is accomplished by modification of standard techniques already described by Shotton et al, 1979; J. Mol. Biol. 131:303-329 and Tyler and Branton, 1980; J. Ultrastruct. Res. 71:95-102. Samples in 0.15M carbonate buffer, pH 7.4, were diluted with glycerol to a final concentration of 70%. Then 100μl of solution were sprayed through an airbrush at an acute angle onto freshly cleaved 6mm mica discs. Droplet sizes were 50-200μm in diameter. Samples were dried in an evaporator at 10^{-6} Torr. Platinum wire was wrapped around the carbon electrodes and the sample was placed on the stage and rotated at 100rpm. At high voltage, the platinum was evaporated to completion at a 6 degree angle from the mica surface. The stage was then tilted 90

degree relative to a carbon source, the chamber was evacuated, and 50Å of carbon were evaporated onto the surface of the mica. The carbon replica was immediately floated off the mica in double-distilled water and mounted onto 400 mesh grids. The samples were examined at 80kV, with a 30μm objective aperture in a transmission electron microscope.

Hybridoma Preparation

Hybridomas were prepared and screened by indirect immunofluorescence as previously described by Sakai et al, 1986; J. Cell Biol. 103:1577-1586. The BM165 mAb, an IgG_1, was purified from cell culture supernatants as described elsewhere (Keene et al, 1990; Collagens XL and XX localize to the surface of banded collagen fibers. J. Cell Biol. in press. Several mAbs were provided by Dr. Eva Engvall of the La Jolla Cancer Research Foundation, and included the mAbs 11D5 (Engvall et al, 1990; Cell Regulation 1:731-740), 4C7 specific for the laminin A chain, (Engvall et al, 1986; J. Cell Biol. 103:2457-2465) and 4E10 specific for the laminin B chain (Wewer et al, 1983; J. Biol. Chem. 258:12654-12660). Rabbit polyclonal antiserum against mouse laminin was obtained from Sigma Chemical Company of St. Louis, Missouri.

Monoclonal antibodies were raised to a mixture of partially purified proteins originally extracted from human amnion by collagenase digestion as described for the isolation of the type VII collagen NC-1 domain (Bächinger et al, 1990; J. Biol. Chem. 265:10095-10101). Resulting hybridomas were screened by indirect immunofluorescence for localization to the dermal-epidermal, but not to the vascular basement membrane zone of human fetal foreskin. Selected hybridomas were rescreened by Western blotting of the immunogen and protein extracts containing known basement membrane components. Hybridomas that did not recognize known basement membrane components were retained for further study. One of these screenings produced two hybridomas that appeared to recognize the same unique protein. One of these, termed BM165, was used for the studies reported here. BM165 specifically identifies the dermal-epidermal junction basement membrane zone of skin, but shows no reactivity to the basement membranes of the vasculature or surrounding nerves (FIG. 1).

The tissue distribution of BM165 reactivity is shown in Table I. All of the subepithelial regions of skin, trachea, esophagus, cornea and amnion showed crisp, brilliant, continuously linear fluorescence. The tissue distribution directly parallels the occurrence of hemidesmosomes and anchoring fibrils, with the exception of the occasional and weak staining of the intestinal smooth muscles. No BM165 reactivity was observed in tissue taken from human kidney, blood vessels, nerve and cartilage.

TABLE I

Tissue Distribution of Antigen Recognized by mAb BM165 As Determined by Indirect Immunofluorescence

Tissue	Result
Skin, subepithelial	+
Trachea, subepithelial	+
Esophagus, subepithelial	+
Cornea, subepithelial	+
Amnion, subepithelial	+
Intestinal smooth muscle	+/-
Kidney	-
Blood vessels	-
Nerve	-
Cartilage	-

The BM165 antibody was then used to localize the antigen within the dermal-epidermal basement membrane of human foreskin. Primary antibody was localized using secondary antibody conjugated to 1nm gold, which was visualized by silver enhancement. The use of 1nm gold was necessary due to the limited penetration of the basement membrane by a 5nm gold conjugated secondary antibody. This procedure localizes the BM165 antigen to the

anchoring filaments, just below the basal dense plate of hemidesmosomes (FIG 2B and 2C). No labeling of the anchoring filaments was seen when an antibody of irrelevant specificity (see Materials and Methods) was employed as the primary antibody (data not shown). Some additional label is seen along the lamina densa (FIG. 2C), but the majority of the label underlies the hemidesmosomes. Small amounts of gold deposits are also seen beneath the dermal side of the lamina densa.

Throughout these experiments, extensive, often complete de-epithelization of skin samples during incubation with the primary antibody was commonly observed. This was entirely outside the inventors' considerable experience with use of antibodies to type IV and VII collagens. The regions of unsplit basement membrane shown in FIGS. 2B and C are those regions that are relatively removed from the tissue edge. Near the tissue edge, where the antibody concentration was highest and the epidermis had separated from the basement membrane, very strong labeling was seen uniformly along the lamina densa, at what had been the cell interface (FIG. 2D). Some label was seen still attached to the extracellular face of the hemidesmosome, but this was relatively rare (not shown).

Antigen Localization

The BM165 antibody was used to visualize the antigen to keratinocyte cultures. As shown in FIG. 3A, when applied to the top surface of a layer of confluent cells, the antibody localizes to the surface of the plastic substrate between the cultured cells (compare to FIG. 3C, which is taken through the cell layer parallel to the culture substrate). No intracellular fluorescence is observed. This unusual localization could not be duplicated with antibodies to type IV collagen (Sakai et al, 1982; Am. J. Pathology 108:310-318), to laminin (see

Materials and Methods) or to type VII collagen (Sakai et al, 1986; J. Cell Biol. 103:1577-1586) (not shown). This localization was not seen when antibodies of the same immunological subtype, but of irrelevant specificity were used (not shown). The antigen is present upon the substrate underneath the cells as shown by strong fluorescence of the entire plastic substrate after removal of the cells with 10mM EDTA (FIG. 3D).

The BM165 antigen is shown in FIG. 4 to immunolocalize along a continuous subcellular matrix in keratinocyte cell culture. Keratinocytes were grown to near confluence and either fixed immediately (FIGS. 4B, 4D) or washed with PBS and incubated with BM165 mAb (50μg/ml) followed by 5nm gold conjugated secondary antibody prior to fixation (FIG. 4A, 4C). Electron microscopic visualization of the antigen in keratinocyte cultures shows linear deposition of immunogold conjugates uniformly across the substrate upon a fine electron dense feltwork (FIG. 4A). The feltwork continued under the cell, but was often unlabeled. Thickenings could occasionally be seen along the keratinocyte plasma membrane that resembled immature hemidesmosomes (FIG. 4B), similar to structures observed by others (Compton et al, 1989; Lab. Invest. 60:600-612).

The ultrastructural immunolocalization studies of BM165 in keratinocyte cultures were complicated by the rounding and detachment of the keratinocytes during long incubations with concentrated BM165 antibody. Scanning microscopy showing the altered morphology of the BM165 incubated cells compared to the morphology of untreated keratinocytes is shown in FIGS. 4C and 4D respectively. The treated keratinocytes in FIG. 4C became rounded and detached during incubation with BM165. Detached keratinocytes readily re-plated onto plastic and grew with equal vigor compared to untreated cells, indicating that

the rounded and detached cells were not metabolically compromised by the antibody treatment (not shown).

To pursue these observations, just subconfluent keratinocyte cultures were incubated with purified BM165 antibody for 10 and 60 minutes, and at each time point the cultures were photographed (FIG. 5A-F). Purified antibody in PBS, PBS alone, or 10mM EDTA were incubated with keratinocytes in parallel. Parallel cultures were also incubated with anti-type VII monoclonal IgG in PBS over the same time course. BM165 antibodies (FIGS. 5C and 5D) and EDTA (FIGS. 5E and 5F) cause extensive rounding and detachment of the keratinocytes by 60 minutes. Such rounding and detachment was not observed when the cultures were incubated with PBS (FIGS. 5A and 5B), anti-type VII collagen nor anti-laminin (not shown). Dermal fibroblasts were rounded and detached by EDTA by not by BM165 (not shown). The BM165 epitope is therefore involved in keratinocyte attachment, but not in the substrate attachment of dermal fibroblasts.

The photomicrographs in FIG. 3A indicate that confluent keratinocyte cultures show no intracellular fluorescence. To evaluate substrate deposition of the antigen occurring relative to the time of plating, keratinocytes were plated at low density, and the development of fluorescence was observed as a function of increasing cell density.

Photomicrographic results of these studies are shown in FIGS. 6A-6E, and demonstrate that synthesis of the BM165 antigen correlates with growing and migrating cells. At 6 hours after plating, only intracellular fluorescence is observed (FIG. 6A). By 24 hours, individual cells and cell clusters can be seen showing both perinuclear intracellular fluorescence and fluorescent staining of the substrate immediately adjacent to the cells (FIGS. 6B, D and E). In some cases, cells

appear to have migrated, leaving behind fluorescent stain attached to the substrate (FIGS. 6D and E). As the cell clusters enlarge (FIG. 6C), only the peripheral cells demonstrate intracellular fluorescence, showing that the cells at the interior of the clusters are no longer synthesizing this antigen. These results are consistent with previous observations that cell growth and migration occur at the periphery of keratinocyte colonies and internal cells are quiescent (Barrandon et al, 1987; Cell 50:1131-1137). They also suggest that the BM165 antigen is produced primarily by the growing and migrating of cells. The interior cells of confluent cultures do not synthesize the antigen.

To further characterize the antigen, the immunogen was fractionated from keratinocyte medium by immunoaffinity chromatography using the BM165 antibody, and was analyzed by polyacrylamide gel electrophoresis (FIG. 7). As described in the Affinity Purification section above, the antigen was affinity purified from spent keratinocyte culture medium. When analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) before disulfide reduction, two species were visualized by staining with Commassie Blue (lane 1). Both molecular species were immunoblot positive (lane 2). The predominant species migrated with an estimated M_r of approximately 400,000, and a minor species of M_r 440,000 was often seen.

Following disulfide bond reduction with mercaptoethanol, four major electrophoretic species were resolved (lane 3, arrows): M_r 165,000,000, 155,000, 140,000 and 105,000. None of these bands are immunoreactive with polyclonal antiserum to EHS laminin (Sigma), or with monoclonal antibodies to human A, B1 or B2 chains (Engvall) (data not shown). Only the 165,000 species (and an immunoreactive smaller species that does

not correspond to any of the chemically stained bands, and is presumed to be a degradation product) contains the BM165 epitope as shown by Western blot when probed with mAb BM165 (lane 4). The disulfide bonded 400kDa and 440kDa species were separately excised from the gel, reduced with 2-mercaptoethanol and the reduction products were separated by electrophoresis. The 400kDa species contains the 165kDa, 155kDa and 140kDa chains (lane 5) and a small amount of a 200kDa species seen only faintly by Commassie stain (lane 3). This 200kDa species also contains the BM165 epitope. The 400kDa species contains the 165kDa, 140kDa and 105kDa chains (lane 7). The results are consistent with identification of a molecule with three non-identical chains. The difference in the electrophoretic migration of the non-reduced species can be explained by a conversion of the 155kDa and to 105kDa by proteolysis. The results also show that the 165kDa chain is related to the 200kDa chain. A precursor product relationship between the 200kDa and the 165kDa chains has been confirmed by biosynthetic pulse-chase experiments. It is not clear if these proteolytic events are physiological.

Rotary shadow imaging of the purified antigen shows a linear molecule containing a central rod of 107nm (FIG. 8). The molecule is seen in two forms. The most common image appears as an extended dumbbell (FIG. 8B) with a globular knob at each terminus of the rod. Often, one knob appears smaller than the other. The least abundant form is asymmetric, with a large globule at one end and two smaller globules at the other (FIG. 8C, the second and third images in that series of three). The images are unlike any previously known to the inventors. The relative abundance of the two images, and the additional knob on the larger species, is consistent with the larger image being contributed by the 440kDa molecule.

Cultured Epidermal Keratinocyte Transplantation

Methods of transplanting keratinocytes have already been disclosed in the literature, and any of these methods are suitable for modification in use with the present invention. Persons skilled in the art would know how such transplants are performed. However, by way of illustration, several examples of suitable transplant methods are disclosed.

EXAMPLE I

One method of keratinocyte transplantation was disclosed by O'Connor et al, 1981; The Lancet 1:75-78. A patient had two $2cm^2$ skin samples taken under local anesthesia. The tissue was placed in culture medium and transferred to a laboratory for cultivation and graft preparation. As much subcutaneous tissue and dermis as possible was removed from the tissue, and the tissue was then minced and trypsinized. The cells were inoculated at different densities (from 10^4 to 10^6 per 50mm dish containing 4×10^5 lethally irradiated 3T3 cells). The cultures were fed with fortified Eagle's medium supplemented with 20% fetal calf serum, hydrocortisone 0.4μg/ml, and choleragen, 0.1nmol/l. The cultures were incubated at 30°C, in an atmosphere containing 10% CO_2. After three days, epidermal growth factor (EGF 10ng/ml) was also added to the culture medium. The medium was changed twice weekly until the cultures either became confluent (between 14 and 21 days) or were subcultured. Some subconfluent cultures were viably frozen and later subcultured. In this way, secondary and tertiary subcultures could be prepared for later use as grafts.

The confluent epithelium was detached intact from the surface of the 50mm tissue culture dish with the enzyme dispase. After detachment, the elastic epithelium shrank to a diameter of 2-2.5cm. It was then washed with

serum-free medium and placed basal side up on two layers of sterile vaseline gauze cut into 2cm circles. Just enough serum-free medium was added to cover the exposed basal surface. Several dishes containing grafts were then placed in a glass jar; the gas phase was flushed with 10% CO_2 and the sealed jar was transported to the bedside.

The epithelial grafts with the vaseline gauze covering were placed on the prepared sites with the basal cell layer directed against the recipient bed. No suturing was necessary because the grafts were held in place by a single layer of non-impregnated fine mesh gauze, which was overlayed with a loose layer of coarse mesh gauze that was changed daily. The fine mesh gauze and the vaseline gauze were removed between the sixth and tenth days and the area was redressed with a single layer of vaseline gauze and a loose layer of coarse gauze. These dressings were changed daily for three to four weeks from the time of grafting. Thereafter, the grafts were left exposed, but treated with a thin layer of lanolin ointment once daily.

The epithelial grafts were placed on three different types of recipient beds: early granulation tissue (less than 7 days old), chronic granulation tissue, and areas recently excised down to the facia.

In accordance with the present invention, adhesion of the confluent epithelium to the underlying tissue would be improved by spreading a thin layer of exogenous kalinin either on the basal face of the keratinocyte culture or on the epithelium of the exposed surface of the tissue on which the graft was being placed. Such exogenous kalinin would provide superior adhesion because the confluent keratinocytes in cell culture have stopped or signifanctly decreased kalinin production, kalinin present upon the basal surface of the cultured cells is destroyed by the dispase treatment, and kalinin is necessary for stabilization of the dermal-epidermal junction.

EXAMPLE II

Methods of grafting autologous cultured human epithelium were also disclosed in Gallico et al, 1984; NEJM 311:448-451. The patients were two children who sustained burns on more than 95% of their bodies, but had half or more of their body surfaces successfully covered with cultured epithelial autografts. On admission, a $2cm^2$ full thickness biopsy specimen of skin was removed from the axilla of each patient. The skin was minced and trypsinized to produce a single cell suspension. Aliquots of 2×10^6 cells were frozen and stored or cultured in flasks with a surface area of $75cm^2$. When the colonies became confluent at 10 days, the cultures were trypsinized, and 3×10^5 cells were inoculated to make secondary and tertiary cultures for grafting. To prepare grafts, the cultured sheets of cells were released from the flasks with dispase, washed with medium, and clipped petrolatum gauze cut to 4.5 x 6 cm. The burn wounds had been excised to muscle fascia, except for third degree facial burns, which were excised tangentially to a depth sufficient to remove dead tissue. The cultured grafts with their gauze backing were placed on prepared wound surfaces, sutured in place, and dressed with dry gauze. The petrolatum gauze was removed 7 to 10 days later. This procedure would be modified, in accordance with the present invention, by amplifying the expression of kalinin by treatment of the released keratinocytes with a cytokine yet to be identified. Since kalinin production appears to be linked to cell proliferation, growth hormones may be possible candidates. Altered feeding schedules might also be effective.

EXAMPLE III

Transplants of autologous cultured human epithelium can be performed as in Examples I and II above.

The method would be modified, however, by transplanting the keratinocytes while a substantial number of them are still actively producing kalinin. In this case, subconfluent keratinocytes would be released from the culture substrate by treatment with 10mM EDTA. The suspended cells are washed with growth medium and suspended in Vitrogen (Collagen Corporation, Palo Alto, California) and poured onto a layer of gauze in teflon forms to produce a thin stabilized layer of single keratinocytes. The Vitrogen would be gelled by brief incubation at 37°C, and the gel would be lifted from the forms and applied to the wound bed. The transferred cells would be protected as in Examples I and II.

EXAMPLE IV

Standard in vitro attachment assays have been performed to determine that purified kalinin facilitates keratinocyte attachment to plastic substrates. In these assays, exogenous purified kalinin or control proteins are incubated overnight with the substrate, and the plates are then washed. Unattached cells are washed away, and the remaining attached cells are quantitated, as described Aumailley et al. (1989); Exp. Cell. Res. 181:463-474.

EXAMPLE V

The role of kalinin in enhancing keratinocyte attachment to a substrate is also demonstrated by treating cell sheets with dispase to release them from a plastic or glass substrate, as would be done in preparing transfer sheets to a wound bed. The sheet is then transferred to a series of plastic substrates which are coated either with kalinin or controlled proteins. The adherence of the sheet is evaluated morphologically to demonstrate that the sheet has superior adherence to the kalinin coated substrate. The adherence of the sheet is evaluated by

indirect immunofluorescence using the BM165 antibody. Firmly attached cell sheets will not allow antibody penetration to the substrate surface as demonstrated by the studies of confluent keratinocyte cultures. Fluorescence beneath the cells would be observed for less firmly attached sheets.

EXAMPLE VI

This example demonstrates the phase of cell culture during which kalinin is synthesized by cultured keratinocytes. Single keratinocytes are plated at various times after culturing begins, and kalinin is localized immunochemically within cells or upon the substrate. Intracellular kalinin is present only within single cells or small clusters of keratinocytes. It is not found intracellularly within the keratinocytes that are in the central regions of large colonies, but only at the periphery where cells are still dividing and migrating.

At various times, cultures are incubated with radioisotopic protein precursors for twelve hours at selected times after plating. Kalinin is then quantitatively immunoprecipitated as a function of total time in culture. The preliminary results of the experiments show that kalinin synthesis decreases with time in culture when measured on a per cell basis (FIG. 10). This information will define the optimal time of cell culture to maximize kalinin production and deposition by keratinocytes.

The present invention includes kalinin from both human and animal sources. Kalinin is present in (and can be purified from) such diverse sources as fetal calf, human amnion and amniotic fluid.

In the future, technical advancements may also permit identification, isolation and purification of individual domains of kalinin which provide keratinocyte adhesion. These domains can be identified by fragmentation of isolated kalinin to produce individual domains, and individualized testing of each domain's ability to function as a keratinocyte attachment factor. Alternatively, domain specific monoclonal antibodies that block cell adhesion could be generated and used to identify the active domain or domains. Once these

advances have taken place, the isolated adhesion domains can be purified and used in the present invention.

Future advances may also permit molecular cloning of kalinin, kalinin sub-chains, or related proteins which provide keratinocyte adhesion. These cloned chains will provide structural information about the identified structural domains. The cloned domains can then be expressed in vitro. If the cell attachment domain is contained within a single kalinin chain, it is possible that a functional fragment could be produced in vitro. Recombinant protein fragments would be transfected into CV-1 cells using the SV40 virus vector as described (Kriegler et al, Gene Transfer and Expression, Stockton Press, New York, 1990).

Summary

The present inventors have isolated a protein from human keratinocyte culture that is involved in the attachment of the cell to plastic in vitro and to the basement membrane in vivo. The identified 165kDa, 155kDa and 140kDa chains apparently form a single molecule with the characteristic rod-like morphology shown by rotary shadowing with two small globules at one end. A second molecule containing the 165kDa, 140kDa and 105kDa fragments is represented by the major rotary shadowed image, lacking the second small globule at one end. These two molecules differ due to cleavage of the 155kDa chain to the 105kDa chain. At this time, it is uncertain whether this proteolytic event is physiological. The inventors have named the new purified protein kalinin which is a word derived from the Greek ξαλινδσ, meaning a bridle or a thong.

The immunolocalization of kalinin to human skin demonstrates that this antigen is the ultrastructural element described as the anchoring filament. The rod-like

shape and the length demonstrated by rotary shadowing of the antigen is also consistent with this assignment. The finding that the majority of the kalinin localizes to the lamina densa following antibody induced rupture of the dermal-epidermal junction suggests that the BM165 antibody epitope lies near the region of the antigen responsible for binding to the hemidesmosome. The opposite end of the antigen appears to be buried in the lamina densa. The observed linear deposition of colloidal gold beneath the basal dense plate shows that this is the site of interaction with the hemidesmosome. When the contact between the hemidesmosome and the anchoring filament is broken by binding of the antibody, the gold seen along the basement membrane is relatively uniformly distributed.

Although the present inventors do not desire to be bound by theories and the limitations of scientific knowledge, the orientation of the molecular elements of the basement membrane zone seen by conventional microscopy may be entirely artifactual. Electron microscopic examination of rat incisor, tongue and gingiva prepared by rapid-freezing and freeze-substitution demonstrate a homogenous 25-100 nm thick electron dense basement membrane completely lacking a lamina lucida (Goldberg et al, 1986; Eur. J. Cell Biol. 42:365-368). The same result has been obtained with the dermal-epidermal junction of human skin by one of the present inventors. Therefore, it is possible that the lamina lucida is an artifact resulting from the cell shrinking away from the basement membrane, and the lamina densa is the residue of the entire basement membrane. If this is the case, it is likely that kalinin is entirely within the basement membrane, with only one end concentrated at the site where the hemidesmosome contacts the basal lamina. The anchoring filaments would then reflect those species within the basal lamina that are strongly bound to the hemidesmosome and become taut and linear as they are

pulled from the basement membrane as the cell shrinks away.

The data also show that in developing or regenerating epithelia, kalinin is initially distributed uniformly upon the migration substrate, and becomes reorganized to the intracellular borders upon maturation of the attachment complex. This is supported by the observation that keratinocytes cultured either on plastic or glass deposit kalinin uniformly upon the substrate, not solely beneath what appear in culture to be immature hemidesmosomes. Once cultures of keratinocytes have become confluent and have a sufficient surface to be grafted on a patient, the confluent culture has stopped depositing kalinin on the substrate. This is believed to account for the poor adhesion of cultured keratinocytes to the dermis, muscle or subcutaneous tissue of a skin graft site.

In view of this observation that kalinin is synthesized only by dividing keratinocytes, it is important to consider the state of confluence of cells to be used for successful re-epithelialization of burn wounds. The results also suggest that kalinin may be deficient or altered in individuals with the blistering conditions such as junctional epidermolysis bullosa (Eady, 1987; Clin. Exp. Dermatol. 12:161-170) or herpes gestationis (Katz et al, 1987; In Dermatology in General Medicine Eds., McGraw Hill, New York, pp. 586-588). Hence, topical application of kalinin may also be useful in treating these conditions, too.

Rotary shadowing of kalinin indicates that it is an asymmetric molecule. This confirmation is consistent with a molecular structure in which one site on kalinin molecules is capable of interaction with receptors on the keratinocyte surface and another part remains buried

within the lamina densa, thus providing cell-substrate adhesion. This impression is further supported by the observed disruption of cell-substrate contact upon incubation of cultured cells with the antibody, and the consistent and dramatic de-epithelization of skin caused by the BM165 antibody.

Having illustrated and described the principles of the invention in several preferred embodiments, it should be apparent to those skilled in the art that the invention can be modified in arrangement and detail without departing from such principles. We claim all modifications coming within the spirit and scope of the following claims.

We claim:

1. Purified kalinin.

2. The purified kalinin of claim 1 which is purified from other basement membrane proteins by immunoaffinity chromatography.

3. The purified protein kalinin that localizes to the hemidesmosomes of basement membranes of human sub-epithelial skin, trachea, esophagus, cornea and amnion, when exposed to monoclonal antibody BM165.

4. The protein of claim 1 wherein the protein is further characterized by providing adhesion between the human dermis and epidermis.

5. The protein of claim 1 wherein the protein has a molecular weight of 400-440kDa and separates on Western blots into fragments of 165kDA, 155kDa and 140kDa when its disulfide bonds are reduced.

6. The protein of claim 1 wherein the protein has a molecular weight of 400-440kDa and separates on Western blots into fragments of 165kDa, 140kDa and 105kDa when it is reduced.

7. The protein of claim 5 wherein the monoclonal antibody BM165 attaches to the 165kDa fragment.

8. The protein of claim 1 wherein a rotary shadow image reveals an asymmetric 170nm long rod including two globules at a first end.

9. The protein of claim 8 wherein the shadow image further includes a single globule at an end of the rod opposite the first end.

10. The protein of claim 1 which on rotary shadow imaging reveals an asymmetric 107nm long rod as shown in FIG. 8.

11. The protein of claim 1 wherein the protein is absent or reduced in the dermal-epidermal junction of humans with junctional epidermolysis bullosa.

12. The purified protein kalinin that localizes to the anchoring filaments of basement membranes of human sub-epithelial skin, trachea, esophagus, cornea and amnion

when exposed to monoclonal antibody BM165, and provides adhesion between human dermis and epidermis, wherein the protein has a molecular weight of approximately 400-440kDa and separates on Western blots into fragments of 165kDa, 155kDa, 140kDa and 105kDa when its disulfide bonds are reduced, the epitope or BM165 is present on the 165kDa fragment, and the protein has the rotary shadow image shown in FIG. 8.

13. A method of improving adhesion of transplanted keratinocytes to an underlying substrate, comprising the step of:

providing an amount of kalinin between the keratinocytes and substrate which is greater than the amount produced naturally by the keratinocytes.

14. The method of claim 12 wherein the step of providing kalinin comprises providing exogenous kalinin.

15. The method of claim 12 wherein the step of providing kalinin comprises increasing the production of kalinin by keratinocytes above basal levels produced naturally by keratinocytes.

16. A method of improving adhesion of transplanted keratinocytes to an underlying substrate, comprising the step of monitoring kalinin production of cell cultures of keratinocytes, and transplanting keratinocytes while they are still actively producing kalinin.

17. The method of claim 13 wherein the substrate is the surface of a burn wound.

18. The method of claim 16 wherein the substrate is the surface of a burn wound.

19. The method of claim 17 wherein the substrate is human dermis or subcutaneous tissue.

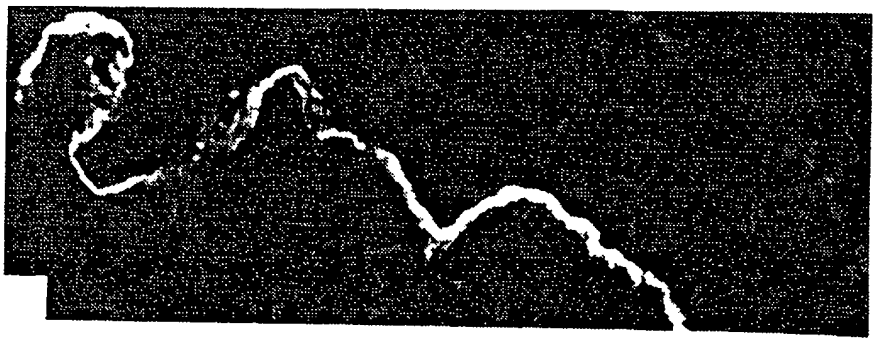

FIG. 1A

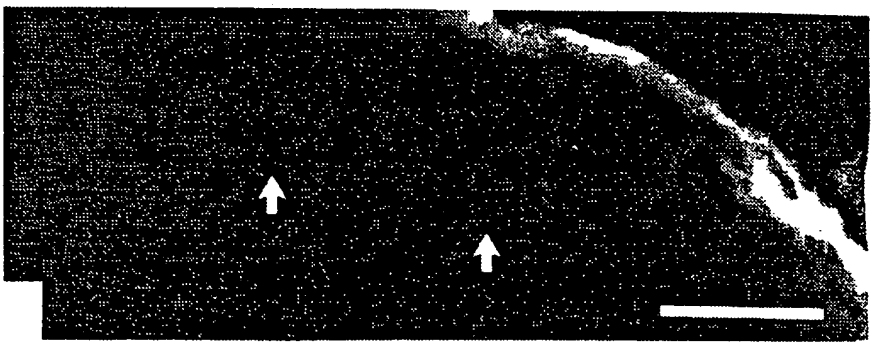

FIG. 1B

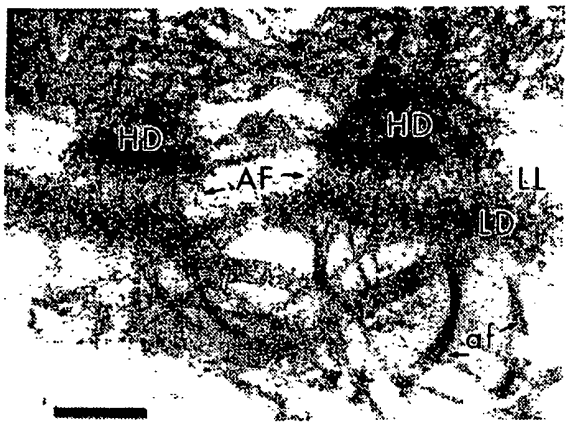

FIG.2A

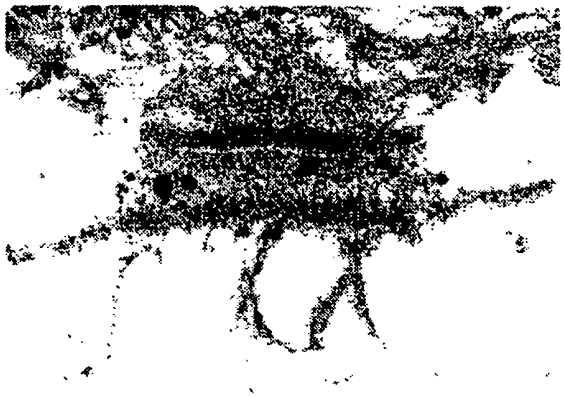

FIG. 2B

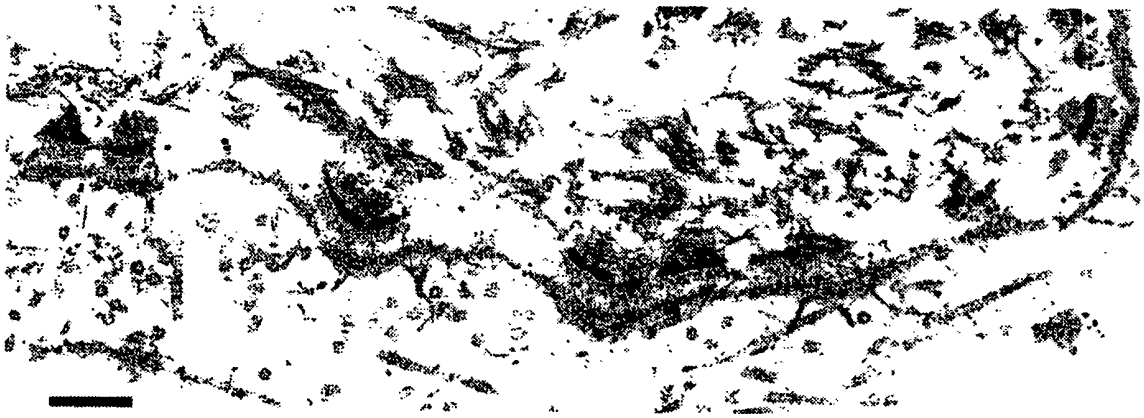

FIG. 2C

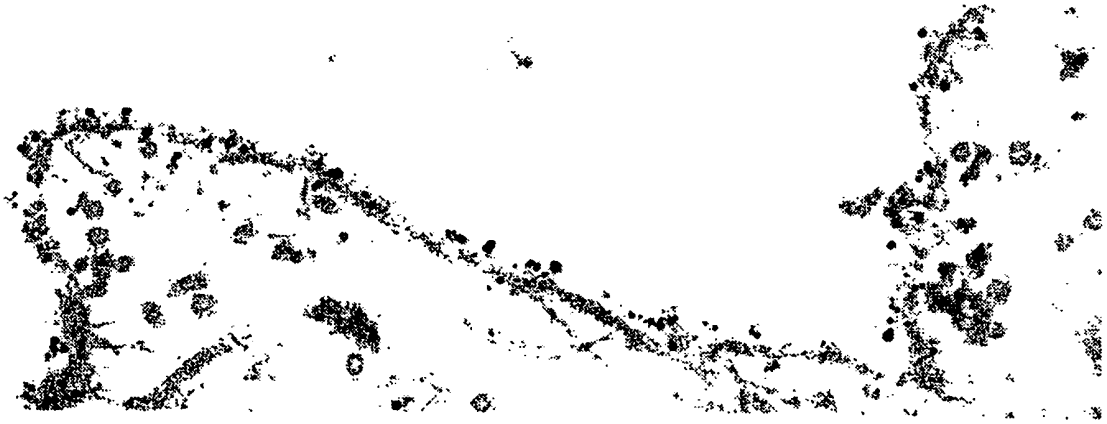

FIG. 2D

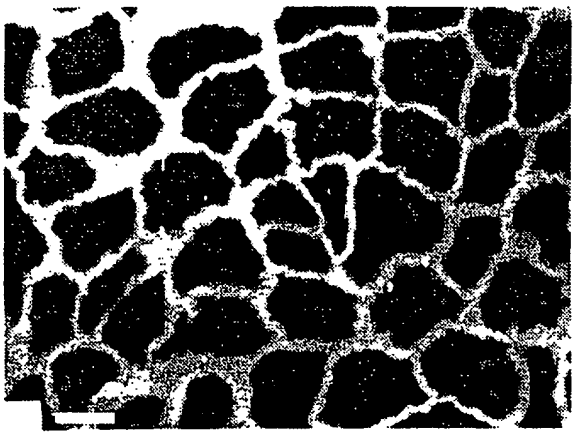

FIG. 3A

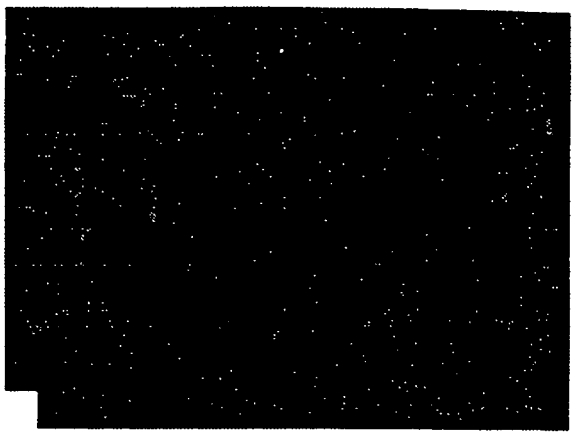

FIG. 3B

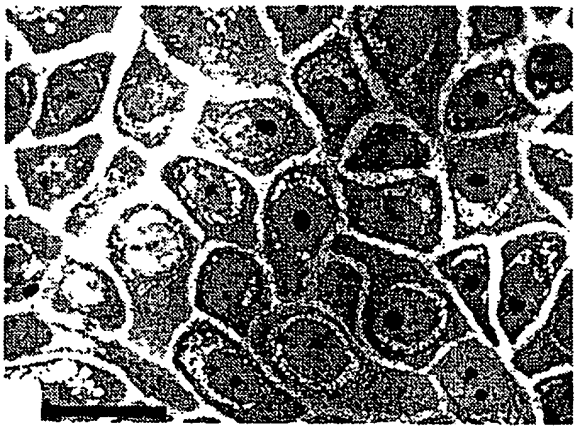

FIG. 3C

FIG. 3D

FIG. 4A

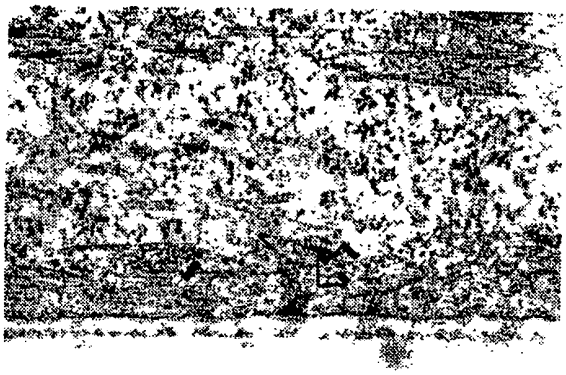

FIG. 4B

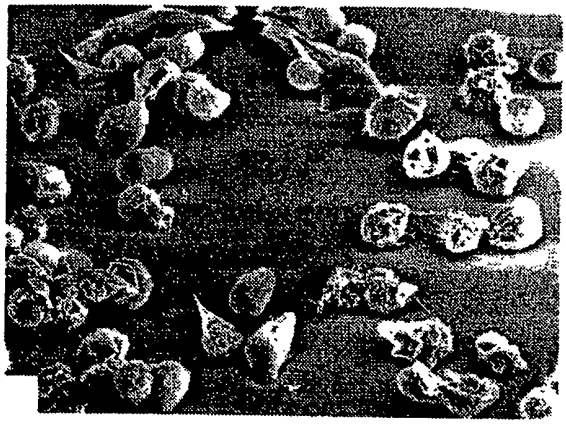

FIG. 4C

FIG. 4D

FIG. 5A

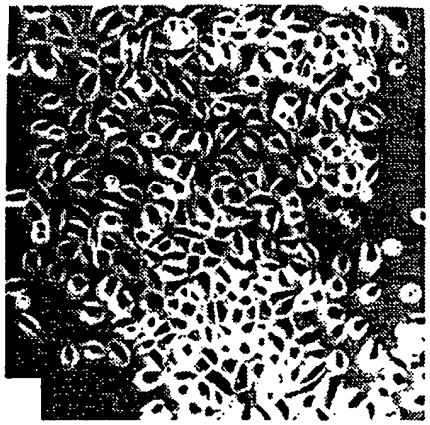

FIG. 5B

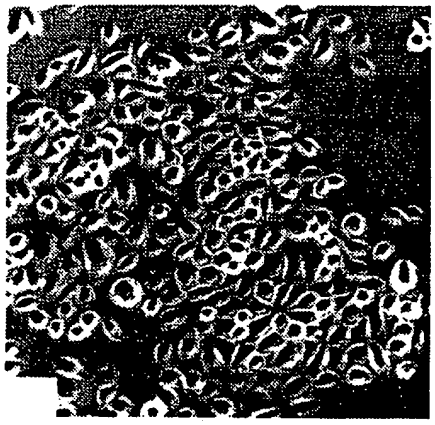

FIG. 5C

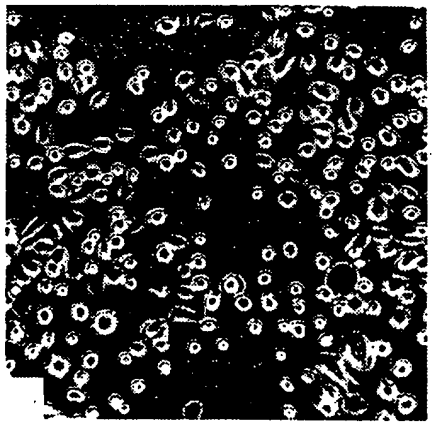

FIG. 5D

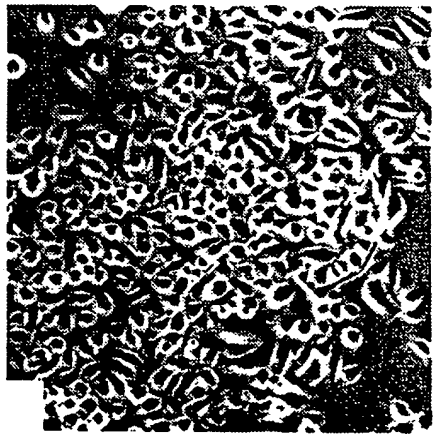

FIG. 5E

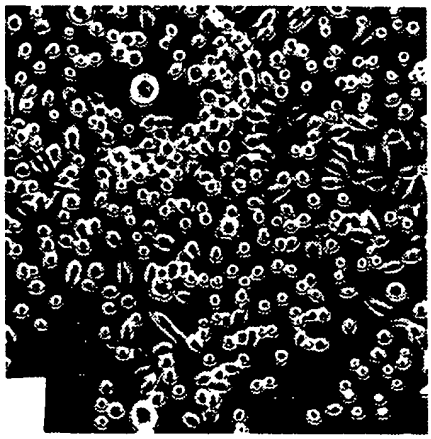

FIG. 5F

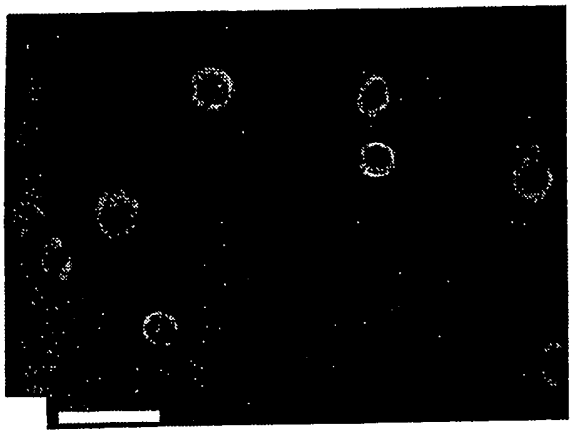

FIG. 6A

FIG. 6B

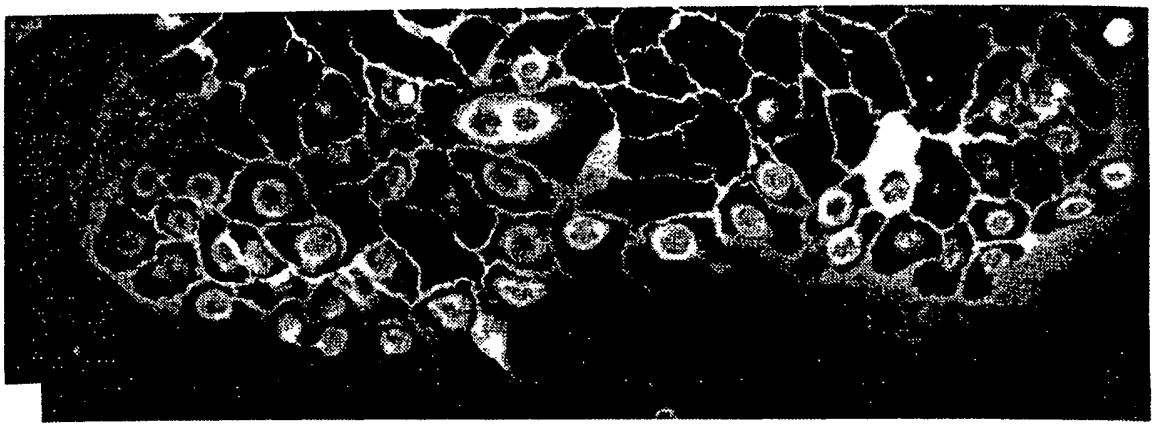

FIG. 6C

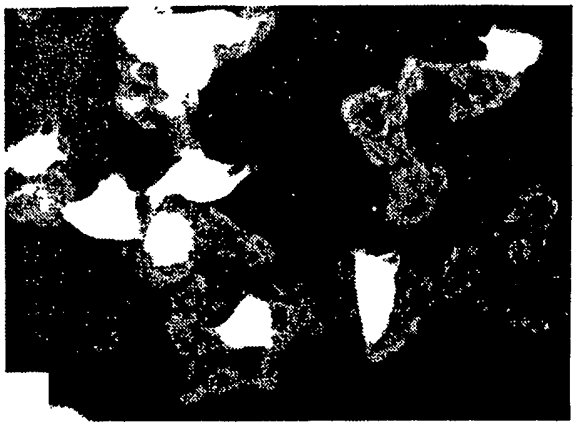

FIG. 6D

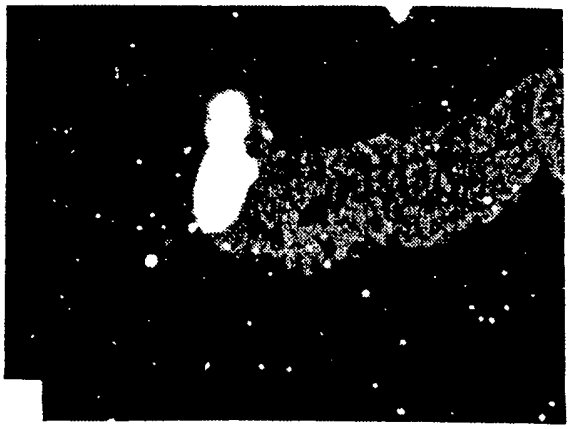

FIG. 6E

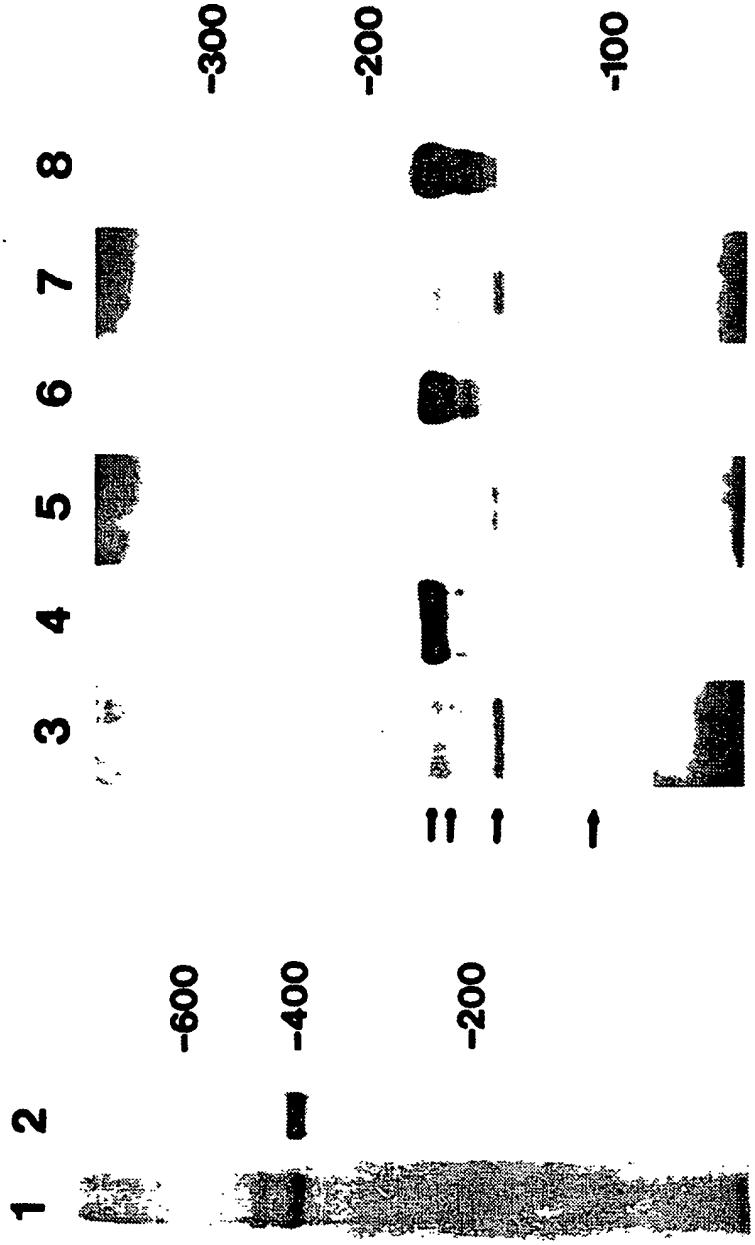

FIG. 7

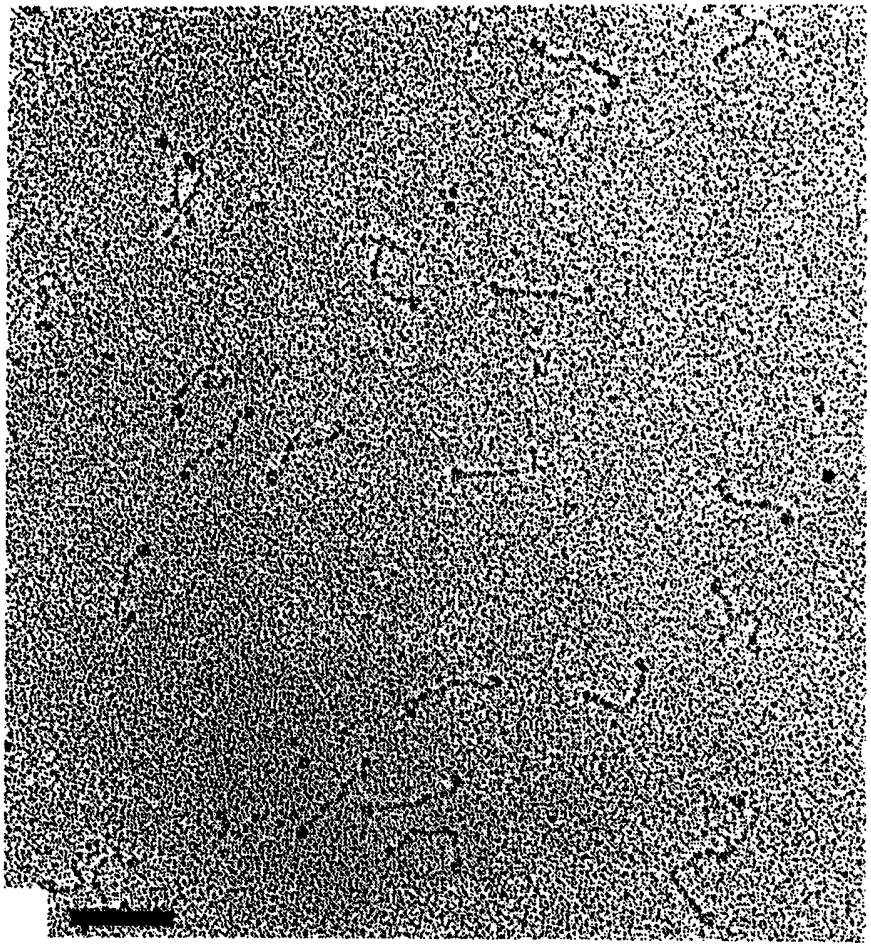

FIG. 8A

FIG. 8B

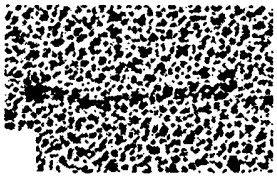

FIG. 8C

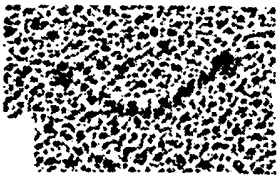

FIG. 8D

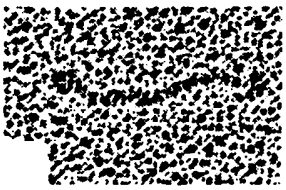

FIG. 8E

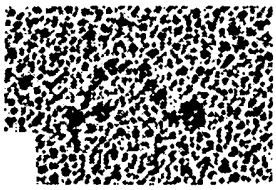

FIG. 8F

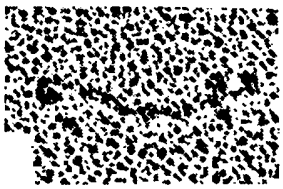

FIG. 8G

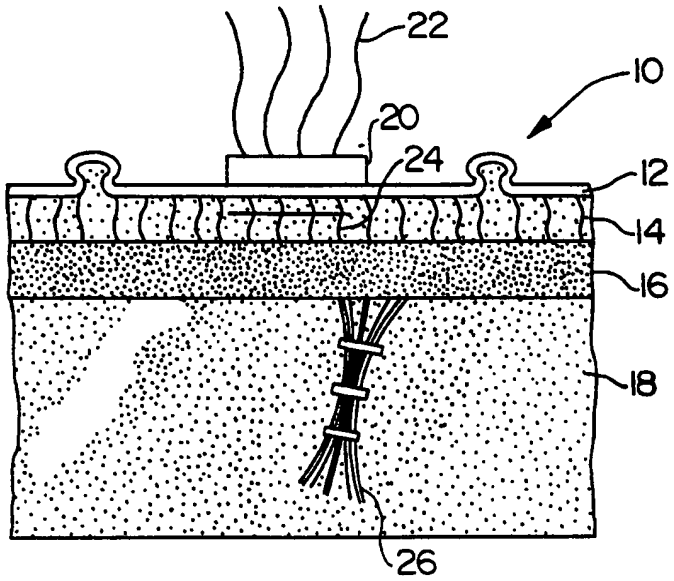
22
10
20
24
12
14
16
18
26

FIG. 9

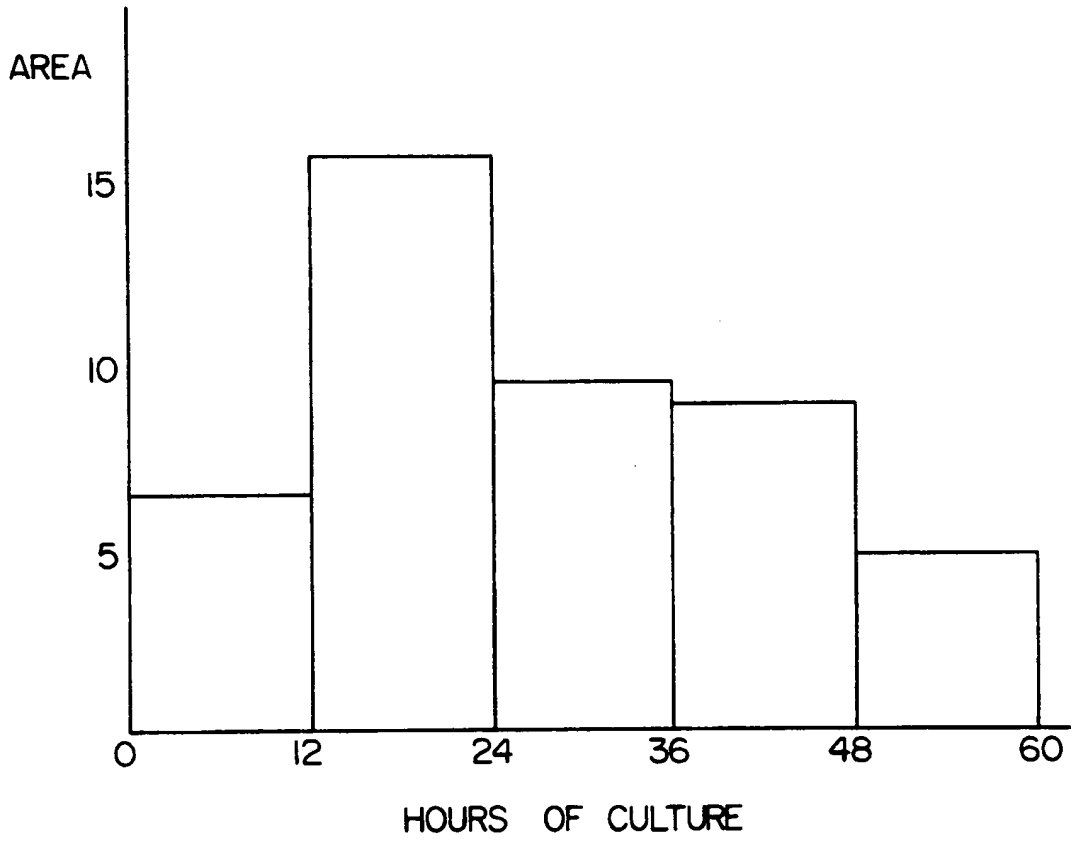
AREA
15
10
5
0
12
24
36
48
60
HOURS OF CULTURE

FIG. 10

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US92/02544

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC (5): C07K 15/00, 15/06, 15/14; A61K 37/02, 37/12
US CL : 530/350, 353, 842; 514/2, 12, 21

II. FIELDS SEARCHED

Minimum Documentation Searched[4]

Classification System	Classification Symbols
U.S.	530/350, 353, 842; 514/2, 12, 21

Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched[5]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[14]

Category*	Citation of Document,[16] with indication, where appropriate, of the relevant passages[17]	Relevant to Claim No.[18]
X	WCTS Meeting Abstract April 26-29, Abstract 5, issued 1990, P. Rousselle et al., "Identification of a New Basement Membrane Antigen", see entire document.	1-12
X	Journal of Cell Biology, Volume 107, Abstract 61, issued October 1989, G.P. Lunstrum, "Identification of New Basement Membrane Antigen which Localizes to the Lamina Lucida and Displays a Novel Tissue Distribution", page 15a, see entire document.	1-12
X	Journal of Cell Biology, Volume 111, Abstract 2231, issued October 1990, D.R. Keene et al., "Kalinin: A Novel Basement Membrane Component Implicated in Keratinocyte Cell Adhesion", page 401a, see entire document.	1-19

* Special categories of cited documents:[16]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search[2]	Date of Mailing of this International Search Report[2]
24 JUNE 1992	30 JUN 1992
International Searching Authority[1]	Signature of Authorized Officer[20]
ISA/US	ANDREW ROZYCKI, EXAMINER GROUP 180

Form PCT/ISA/210 (second sheet)(May 1986) ß

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

X	Experimental Cell Research, Volume 170, issued 1987, P. Verrando et al., "Monoclonal Antibody GB3, A New Probe for the Study of Human Basement Membranes and Hemidesmosomes", pages 116-128, see pages 126-127.	1-12
X/Y	Biochimica et Biophysica Acta, Volume 942, issued 1988, P. Verrando et al., "The New Basement Membrane Antigen Recognized by the Monoclonal Antibody GB3 is a Large Size Glycoprotein: Modulation of its Expression by Retinoic Acid", pages 45-56, see pages 48-50, 54-56.	1-12/13-19

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE[1]

1. ☐ Claim numbers _, because they relate to subject matter (1) not required to be searched by this Authority, namely:

2. ☐ Claim numbers _, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out (1), specifically:

3. ☐ Claim numbers _, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING[2]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Search Authority did not invite payment of any additional fee.

Remark on protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category*	Citation of Document,[16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No.[18]
Y	BMJ, Volume 299, issued 11 November 1989, K. Hancock, "Cultured Keratinocytes and Keratinocyte Grafts", pages 1179-1180, see entire document.	13-19
A	US, A, 4,829,000 (Kleinman et al) 09 May 1989, see entire document.	1-13
A	Journal of Investigative Dermatology, Volume 77, No.3, issued 1981, O. Saksela et al., "Basal Lamina Components in Experimentally Induced Skin Blisters", pages 283-286, see entire document.	1-13

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